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The Effects of Pesticides on Some Biochemical Parameters of Carp (*Cyprinus carpio* L.)

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Abstract. Treatments with copper sulphate ($CuSO_4$), paraquat (PQ) and methidathion (MD) caused tissue damage and stress effects in carp, indicated by the increased lactate dehydrogenase (LDH), glutamic oxaloacetic transaminase (GOT), and glutamate dehydrogenase (GlDH) enzyme activities and elevated blood-sugar levels.

Copper sulphate, administered together with PQ and MD, were synergistic in terms of tissue damage and stress effects.

The isoenzyme patterns showed organ-specific tissue damage. The administered chemical and isoenzymes indicating liver damage were detectable in the blood.

The combination of $CuSO_4$ and MD caused focal cell necrosis, which was observable in the liver tissue by light microscopy. Electron microscopic studies revealed the presence of damaged parenchymal cells with electron transparent cytoplasms, myelin figures, and altered mitochondria ER and Golgi.

The off-target movement of chemicals used in industry and agriculture is usually unavoidable. These chemicals get into natural water and may cause significant tissue damage in fish (McKim *et al.* 1970; Reichenbach-Klinke 1972; Horváth and Stammer 1979; Ferri and Macha 1980; Rojik *et al.* 1983; Benedeczky *et al.* 1984). The degree of increase in activity of the cellular enzymes in sera depends primarily on the magnitude and severity of cell damage (Schmidt and Schmidt 1976; Kristoffersson *et al.* 1974; Nemcsók *et al.* 1981; Nemcsók and Boross 1982). Furthermore pollutants may get into water in combination with each other, causing additive harmful effects on the fish. The changes in the enzyme activities of LDH, GlDH, GOT have been used for demonstrating tissue damage in fish (Wrobelski and La Due 1955; Kristoffersson *et al.* 1974; Nemcsók and Boross 1982; Asztalos and Nemcsók 1985). Also, the increase in the blood glucose level has been used for demonstrating metabolic stress (Wedemeyer 1970; Nemcsók and Boross 1982). The magnitude of the increase in LDH in the blood sera shows the degree of tissue damage and the LDH isoenzyme pattern reflects which tissue are damaged (Herbert *et al.* 1970; Asztalos and Nemcsók 1985). LDH isoenzymes are suitable tools for the indentification of damaged organs in human clinical diagnosis (Fogh-Andersen 1982). Two types of LDH isoenzymes are found in mammals: M subunits are characteristic of the skeletal muscle and H subunits are characteristic of the heart muscle (Appela and Markert 1961). The so-called type C isoenzyme can also be found in fish, specific to the liver (Markert and Faulhaber, 1965; Shaklee *et al.* 1973; Goldberg, 1966). Analysis of LDH isoenzyme pattern in blood sera of carp revealed which tissue organ necrosis is responsible for the increased LDH activity caused by pesticides (Asztalos and Nemcsók, 1985). Since pesticides may occur as potentially toxic chemicals in fish ponds and rivers, in this work the effects of copper sulphate (fungicide), methidathion (O,O-dimethyl-S)2methoxy-1,3,4-thiadiazol-5(4)onyl-4-methyl(dithiophosphate) (insecticide), paraquat (1,1′-dimethyl-4,4′-bipyridynium dichloride) (herbicide) on the activity of LDH, GlDH, GOT and blood glucose levels in carp sera were examined as well as the LDH isoenzyme pattern after each treatment, singly and in combination. In addition to the biochemical measurements, a detailed light- and electronmicroscopic investigation was carried out on the liver in order to identify the cytopathological effect of the pesticides in the liver.

Materials and Methods

Carp (*Cyprinus carpio* L.) of both sexes weighing 1,000-1,200 g were used in the experiments. The fish were kept in a 100-L aquarium in O_2-saturated water. The animals were not fed during the experiments. The temperature of the water was 10 ± 1°C. Hungarian data indicate that in pesticide polluted water chemicals may accumulate in different fish organs (muscle, fatty tissue) up 10 ppm. Therefore pesticide concentrations used were as follows: $CuSO_4$ 5 mg/L; paraquat[1] 5 mg/L; methidathion[1] 2 mg/L; in cases of combined exposure, half the above quantities were used. The duration of the treatments with each compound was 1, 4, 6, and 14 days.

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[1] Paraquat = 4,4′-Bipyridinium, 1,1′-dimethyl dichloride; Methidathion = *S*-[(5-methoxy-2-oxo-1,3,4-thiadazol-3(2*H*)-yl)methyl] *O,O*-dimethyl phosphorodithioate.

Samples were taken from the treated and control fish before and at each designated time point. Blood samples were centrifuged and changes in GOT, LDH, GlDH activities and blood glucose level were measured in the non-haemolyzed sera. Results are expressed as a percentage of the pretreatment and control activity. The values are expressed as the average of 6–18 individuals (±S.E.M.).

Determination of GOT Activity (Reitman and Frankel 1957)

Reaction mixture for GOT: 0.25 ml pH 7.4 phosphate buffer containing 0.1M L-aspartate and 2 mM α-ketoglutarate + 0.05 ml blood serum (0.05 ml distilled water in blank). After 60 min. incubation at 37°C, 0.25 ml 1 mM 2,4-dinitrophenylhydrazine was added to each sample and the mixture was incubated for 20 min. at 20°C. After the addition of 2.5 ml 0.4 M NaOH solution, the absorbance was measured at 546 nm.

Determination of LDH Activity (Annon 1971)

Reaction mixture: 3 ml of 52 mM pH 7.5 phosphate buffer containing 0.63 mM pyruvate + 0.05 ml 11 mM NADH + 0.1 ml blood serum. Read absorbance at 340 nm within 30 sec after serum was added. Repeated reading after exactly 1, 2, and 3 min.

Determination of Blood Glucose Level

Reaction mixture: 2.5 ml glucose reagent 2 mg peroxidase + 12.5 mg glucose oxidase diluted in 50 ml 0.1 M pH 7.0 phosphate buffer + 3.3 mg *o*-dianisidin diluted in 1 ml distilled water + 0.1 ml deproteinized blood serum. After 35 min. incubation at room temperature. Absorbance was measured at 450 nm.

Determination of GlDH Activity (Schmidt and Schmidt 1983)

For the determinations, Boehringer and Reanal KIT-s (Hungary) were used. Biochemical assays were performed by spectrophotometric methods, using Varian (Australia) Spectrophotometer. Cell path length 1 cm.

For the determination of LDH isoenzymes, the separated organs (heart, muscle, skeletal muscle, liver) were homogenized in a glass tube in a five-fold volume of ice-cold 52 mM phosphate buffer, pH 7.5 for LDH. Each homogenate was centrifuged at 12,000 g for 3 min. at 4°C. The supernatant was used for the determination of the enzyme activity and isoenzyme pattern. LDH activity in blood was measured spectrophotometrically by monitoring the decrease of NADH (pyruvate reduction). LDH isoenzyme patterns were determined by electrophoresis, using a vertical slab of polyacrylamide 4-6% gradient gel containing 3% *N,N'*-methylene-bis-acrylamide (Goldberg 1966; Asztalos and Nemcsók 1985). The gelling accelerator was ammonium persulphate. The gel buffer was 1.32 M Tris-HCl, pH 8.9. The electrode buffer was 0.5 M Tris-glycine pH 8.3. The composition of the staining solution was as follows: 1.3 mg NAD, 0.45 mg NBT (*P*-nitro-tetrazolium blue chloride), 0.14 mg PMS (phenasine methosulphate) in 1 ml Tri-HCl buffer, pH 7.6. The electrophoresis was accomplished at 4°C at a constant current (3 mA/mm²) for 2.5 hr. Sample volumes were 25–50 µl.

For electron microscopic investigations small slices were cut from various areas of the liver with a sharp safety razor, and the slices were immediately placed into ice-cold fixative. 2.5% glutaraldehyde was used for prefixation, buffer to pH 7.3 with cacodylate buffer. Following 4 hr prefixation, the samples were fixed in 2% OsO_4 solution for an additional 2 hr. After dehydration in graded alcohol (30; 50; 75; 96; 100%), 75% ethanol was saturated with uranyl acetate to stain the blocks in which the samples were embedded (Durcupan ACM, Fluka). The ultrathin sections were contrasted with lead and studied in the electronmicroscope.

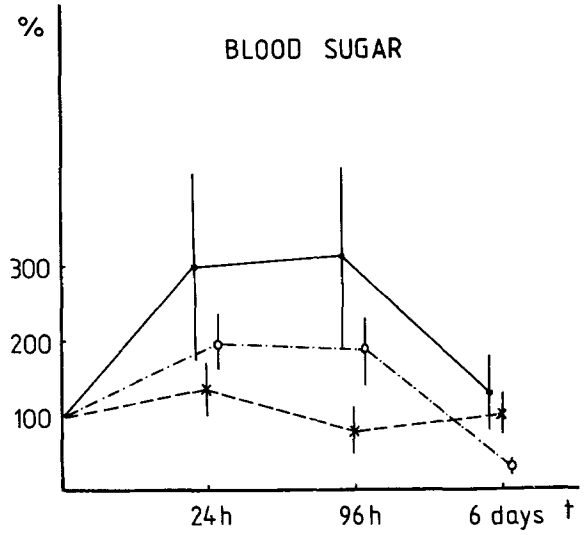

Fig. 1. The effect of 5 mg/L $CuSO_4$ (--●--), (n = 6–18); 2 mg/L MD (--×--), (n = 6–18); 2.5 mg/L $CuSO_4$ + 1 mg/L MD (--○--), (n = 6–18) on the blood glucose level of carp over the exposure time. The values are expressed as the average of 6–18 individuals. Water temperature 10°C ± 1°C

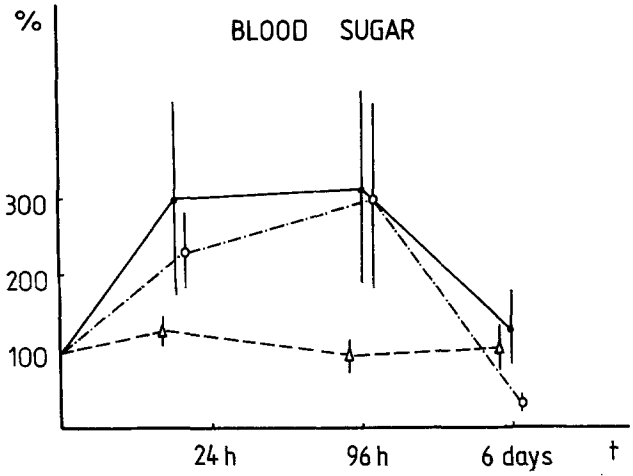

Fig. 2. The effect of 5 mg/L $CuSO_4$ (--●--), 5 mg/L PQ (--△--), 2.5 mg/L $CuSO_4$ + 2.5 mg/L PQ (--○--) on the blood glucose level of carp over the exposure time. The values are expressed as the average of 6–18 individuals. Water temperature 10°C ± 1°C

Results and Discussion

Blood Sugar

Following $CuSO_4$ treatment (5 mg/L) the blood sugar level significantly increased on the first day, remained elevated for three subsequent days, then decreased, beginning on the fourth day (Figures 1, 2).

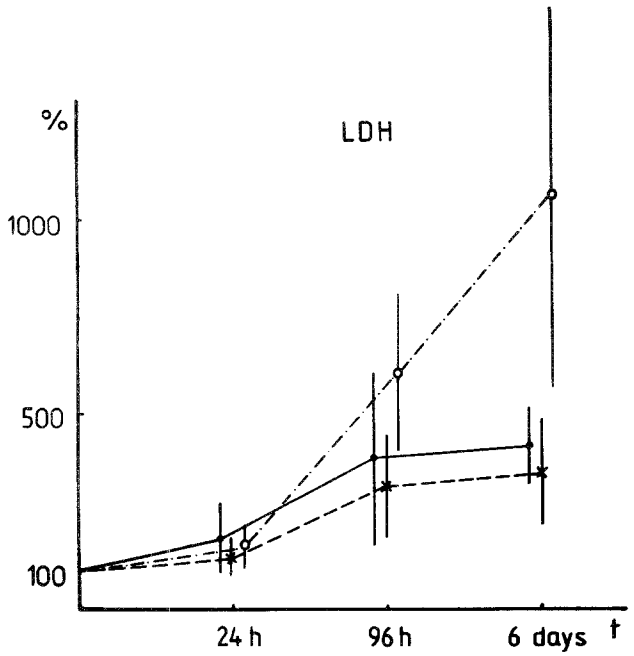

Fig. 3. The effect of 5 mg/L $CuSO_4$ (--●--), 2 mg/L MD (--×--), 2.5 mg/L $CuSO_4$ + 1 mg/L MD (--○--) on the serum LDH activity of carp over the exposure time. The values are expressed as the average of 6–18 individuals. Water temperature 10°C ± 1°C

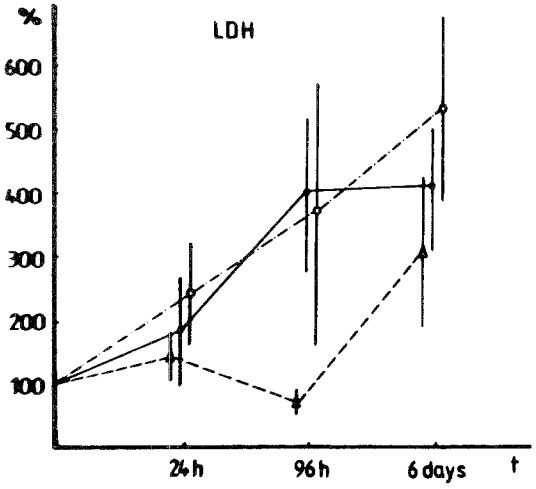

Fig. 4. The effect of 5 mg/L $CuSO_4$ (--●--), 5 mg/L PQ (--▲--), 2.5 mg/L $CuSO_4$ + 2.5 mg/L PQ (--○--) on the serum LDH activity of carp over the exposure time. The values are expressed as the average of 6–18 individuals. Water temperature 10°C ± 1°C

Considerable changes were manifested in the blood-sugar level (Figure 1). Experiments with rats and mice revealed that the organism is in need of NADPH for the *in vivo* decomposition of MD (Chopade and Dauterman, 1981), meaning that the MD enhances the direct oxidation of glucose through NADP produced in the course of detoxication. This may explain why glucose does not appear in the blood of MD-treated animals in large quantities. The blood-sugar level remained at the control value after PQ treatment at a dose of 5 mg/L (Figure 2). The unchanged blood-sugar value in PQ-treated fish does not necessarily mean PQ did not cause stress in the fish, since PQ also enhances glucose decomposition by means of the pentose-phosphate cycle (Rose *et al.* 1976) and thus the blood-sugar released due to stress may be utilized by the pentose-phosphate pathway. The combination of MD or PQ with $CuSO_4$ (2.5 mg/L PQ + 2.5 mg/L $CuSO_4$, or 1 mg/L MD + 2.5 mg/L $CuSO_4$) caused changes in blood-sugar levels proportional to the lower $CuSO_4$ concentration (Figures 1, 2). This may indicate that in the combined treatments, $CuSO_4$ alone is responsible for the elevation of the blood-sugar level. The fact that the blood-sugar levels decreased to 30% of the control value on the sixth day is indicative of the exhausted glucose-reserves of the organism. This may reflect a continuous stress effect due to the treatments (Vig *et al.* 1987), or it may be due to the damage to the branchial epithelium (Rojik *et al.* 1983; Benedeczky *et al.* 1986). The process of sugar decomposition shifted towards the anaerobic direction, energetically being quite unfavorable (Rojik *et al.* 1983).

LDH

Treatment with 5 mg/L $CuSO_4$ caused an increase in the LDH enzyme activity in the blood serum until the fourth day, which then stayed at this value (Figures 3, 4). Following 2 mg/L MD treatment, similar enzyme activity increase was manifested (Figure 3). After PQ treatment in a 5 mg/L dose, the two-step emptying of PQ is reflected by the change in the serum LDH activity. On the first day following treatment, a weak change in LDH level was manifested, while a three-fold change was experienced on the sixth day (Figure 4). This may indicate that PQ had a damaging effect on the heart muscle, besides the inhibition of acetylcholinesterase (Nemcsók *et al.* 1984) as we earlier demonstrated. PQ in the blood shows a weak binding to mammalian plasma proteins (Conning *et al.* 1969), circulating partially in free form and thus being able to reach the tissues rapidly. In the Conning (1969) study, a large part of the PQ incorporated in the tissues empties within 48 hr and causes only slight pulmonar tissue damage. However, 20–30% of the PQ remains in the tissues and is only evacuated after 2–3 weeks (Fischer *et al.* 1971). This portion staying in the tissues for a long time causes significant tissue damage, which is observable by the sixth day in this study as a strong increase in the blood LDH level. $CuSO_4$, administered together with either PQ or MD, acts as a synergist in the development of tissue damage, as indicated by the extremely high LDH activity in the blood (Figures 3, 4). On the basis of the blood LDH isoenzyme pattern, the organ from which the LDH originated can be determined indicating which organ's cells became damaged. From LDH isoenzyme patterns conclusions can also be drawn with respect to what tissues incorporate the pesticides as well as which ones are primary target sites (Asztalos and Nemcsók 1985).

GOT

The GOT enzyme activity which increased in the blood following combined treatments may also indicate liver injury (Figures 5, 6), as has been demonstrated in salmon (Bell 1968) and pike (Kristoffersson *et al.* 1974). Following 5 mg/L PQ and 2.5 mg/L PQ + 2.5 mg/L $CuSO_4$ treatments, the

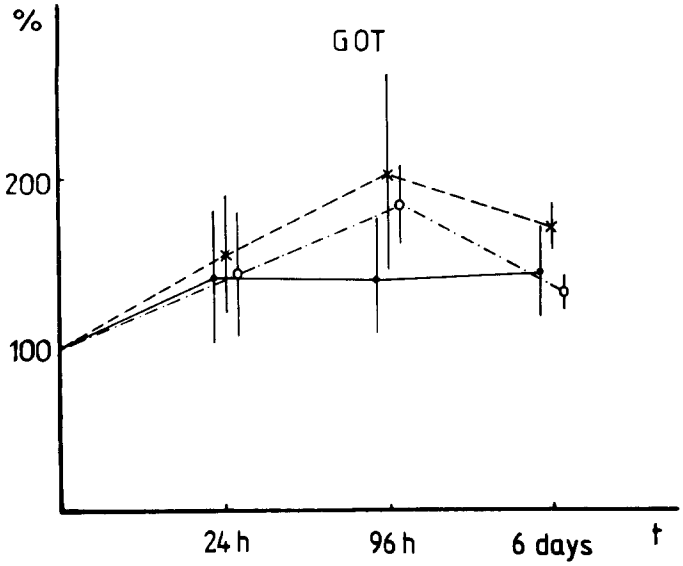

Fig. 5. The effect of 5 mg/L $CuSO_4$ (--●--), 2 mg/L MD (--×--), 2.5 mg/L $CuSO_4$ + 1 mg/L MD (--○--) on the serum GOT activity of carp over the exposure time. The values are expressed as the average of 6–18 individuals. Water temperature 10°C ± 1°C

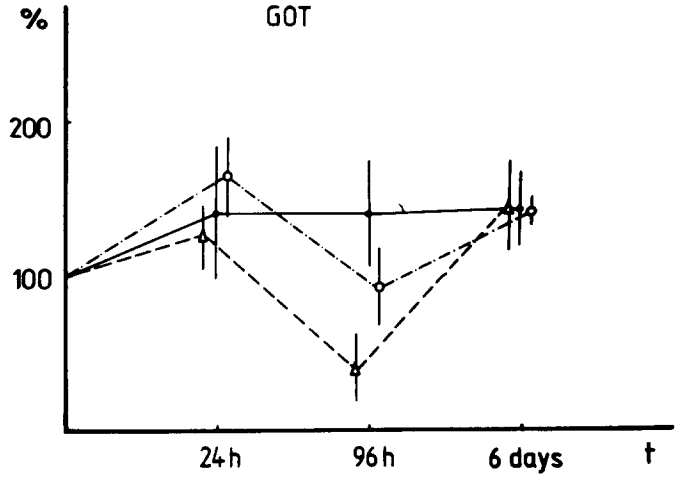

Fig. 6. The effect of 5 mg/L $CuSO_4$ (--●--), 5 mg/L PQ (--△--), 2.5 mg/L $CuSO_4$ + 2.5 mg/L PQ (--○--) on the serum GOT activity of carp over the exposure time. The values are expressed as the average of 6–18 individuals. Water temperature 10°C ± 1°C

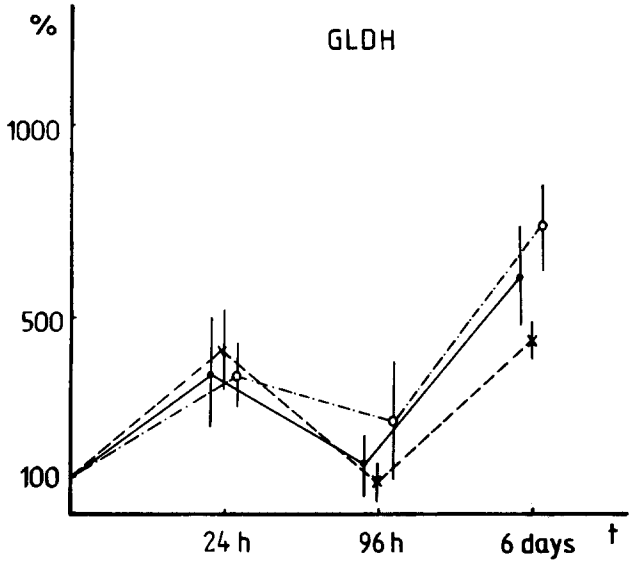

Fig. 7. The effect of 5 mg/L $CuSO_4$ (--●--), 2 mg/L MD (--×--), 2.5 mg/L $CuSO_4$ + 1 mg/L MD (--○--) on the serum GlDH activity of carp over the exposure time. The values are expressed as the average of 6–18 individuals. Water temperature 10°C ± 1°C

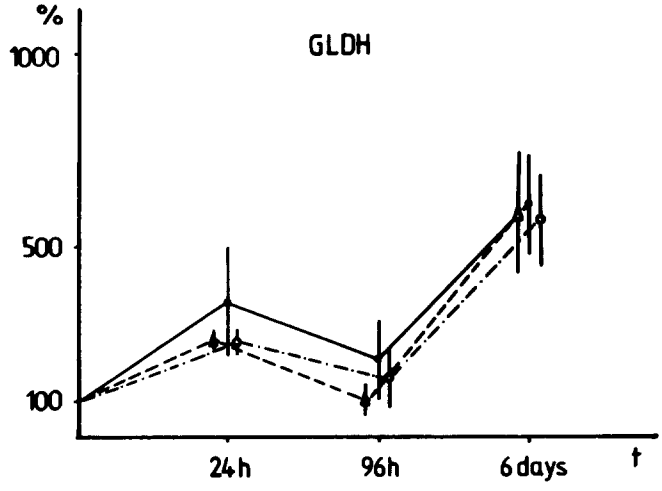

Fig. 8. The effect of 5 mg/L $CuSO_4$ (--●--), 5 mg/L PQ (--△--), 2.5 mg/L $CuSO_4$ + 2.5 mg/L PQ (--○--) on the serum GlDH activity of carp over the exposure time. The values are expressed as the average of 6–18 individuals. Water temperature 10°C ± 1°C

increase of the GOT enzyme activity in the blood reflects the two-step evacuation of PQ (Figure 6). During the course of the PQ-$CuSO_4$ combined treatment, both agents are responsible for the change in GOT enzyme activity while in the case of 2.5 mg/L $CuSO_4$ + 1 mg/L MD treatment, this change is moderated by MD. The cause of the decrease in GOT activity in the blood starting from the fourth day could be that the damaged hepatocytes are no longer capable of synthesizing the GOT protein.

GlDH

Following single Cu, MD, PQ, and both $CuSO_4$-MD and $CuSO_4$-PQ combined treatments (Figures 7, 8), the GlDH enzyme activity of the blood serum falls to the control value on the fourth day after the initial rise, then increases again vigorously. The increase of the GlDH enzyme activity in the blood may be due to the exhaustion of its glucose reserves; the organism tries to ensure the necessary energy demand by protein decomposition. The increased GlDH activity may be involved in the detoxication of ammonia formed during protein degradation. Such biochemical alterations may occur in many fish species in relation to various pesticides. The application of this biochemical measurement, however, would serve to alert an investigator to the presence of a pesticide in the aquatic environment. On the basis of the obtained results, the conclusion could be drawn that it is worthwhile to study the common damaging effect of the chemicals—by means of biochemical methods—occurring as contaminants exerted on the living organism, in order to reveal the possible synergistic antagonistic phenomena.

Following $CuSO_4$ treatment, isoenzymes characteristic of heart muscles were demonstrable in the blood (Figure 9).

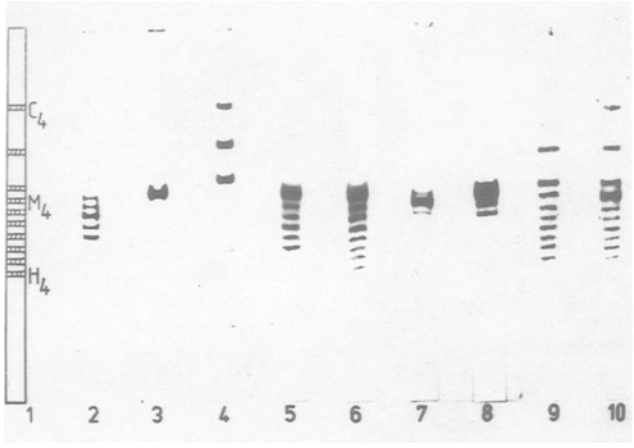

Fig. 9. LDH zymograms of blood sera of carp: (1) diagrams of zymograms of LDH in fish, (2) control heart muscle, (3) control skeletal muscle, (4) control liver, (5) blood sera after 5 mg/L PQ treatment for 6 days, (6) blood sera after 5 mg/L $CuSO_4$ treatment after 6 days, (7) blood sera after 2.5 mg/L $CuSO_4$ + 1 mg/L MD treatment after 6 hr; enzyme activity 400 U/L, (8) blood sera after 2.5 mg/L $CuSO_4$ + 1 mg/L MD treatment after 26 hr; enzyme activity 600 U/L, (9) blood sera after 2.5 mg/L $CuSO_4$ + 1 mg/L MD treatment after 4 days; enzyme activity 2400 U/L, (10) blood sera after 2.5 mg/L $CuSO_4$ + 1 mg/L MD treatment after 6 days; enzyme activity 2540 U/L. Columns 7–10 represent the same treatment with different exposure times

Table 1. Mortality of carp after chemical treatment

Treatment	Survival %		
	1st day	4th day	6th day
5 mg/L PQ[a]	100	100	100
5 mg/L $CuSO_4$	100	61	44
2 mg/L MD[b]	100	72	66
2.5 mg/L $CuSO_4$ 2.5 mg/L PQ	100	83	83
2.5 mg/L $CuSO_4$ 1.0 mg/L MD	100	44	31

[a] PQ = Paraquat
[b] MD = Methidathion

This is in agreement with the experiments of Nemcsók *et al.* (1987) performed with isotopic copper sulphate, during the course of which the copper was found mainly to incorporate into heart and skeletal muscle. Damage to heart muscle was also detectable due to PQ treatment (Figure 9 column 5) as it is represented by isoenzymes characteristic for control heart muscle (Figure 9 column 2).

In a previous experiment, (Asztalos and Nemcsók 1985) damage was found mainly in the gill of carp after PQ treatment. In the current experiment, the development of gill damage can not be excluded, due to the similarity of heart and gill isoenzymes. A significant heart muscle injury may conceal the isoenzymes characteristic to the gill. Following 2 mg/L MD treatment, enzymes indicating damage to the skeletal muscle were found in the blood of carp (Asztalos and Nemcsók 1985). In this experiment, treatment with 2.5 mg/L $CuSO_4$ + 1 mg/L MD yielded various isoenzyme patterns in the blood, depending on the degree of the LDH enzyme activity present in the blood (Figure 9):

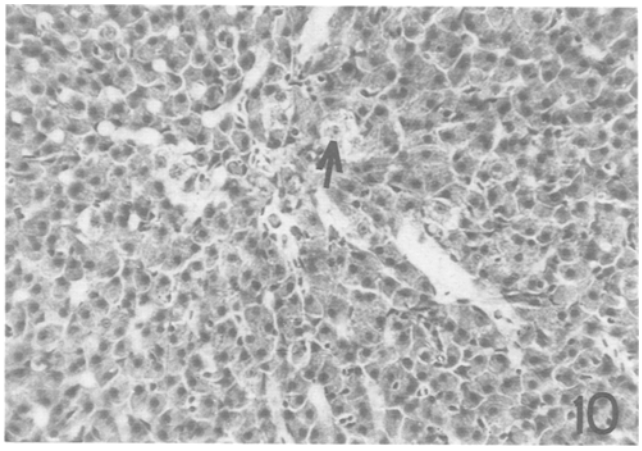

Fig. 10. Light microscopic picture of carp liver, two weeks after $CuSO_4$ + MD treatment. Note detailed extracellular spaces, vacuoles and focal cell necrosis (arrow). 160×

1. In case of low LDH enzyme activity the isoenzymes characteristic to the skeletal muscle appeared in the blood on the sixth day (see columns 3 and 7). This can be explained by the fact that the decomposition of MD takes place rapidly (Visi 1984); thus, the agent exerts its effect in the various organs for only a short time, and presumably only the tissue with the greatest bulk (skeletal muscle) is capable of releasing enough LDH into the blood, to be stainable on polyacrylamide gel (Schmidt and Schmidt 1976).
2. Somewhat higher LDH enzyme activity (in the $CuSO_4$ + MD treatment) displays isoenzymes in the blood characteristic of heart muscle (see columns 2, 8, 9). The reason for this may be that $CuSO_4$ is eliminated from the heart slowly, causing damage to the heart muscle with time.
3. In the case of extremely high LDH enzyme activity (in the $CuSO_4$ + MD treatment), isoenzymes characteristic of skeletal muscle, heart muscle, and liver tissue appeared in the blood, by the fourth and sixth days (see columns 2, 3, 4, and 10).

Treatments which caused liver injury as demonstrated by the presence of LDH "C" isoenzyme in the blood, also caused considerable mortality (Table I). Unambiguous signs of liver injury are observable on the light—and electron-microscopic figures as well (Figures 10, 11, 12). Two weeks after 2.5 mg/L $CuSO_4$ + 1 mg/L MD treatment focal cell necrosis could be observed by light microscopy (Figure 10). By electron microscopy the endoplasmic matrix showed light, electron-lucent character in the majority of the hepatocytes (Figure 11). The present of light, cell organelle-free cytoplasmic fields are presumably related to the disappearance of the glycogen granules and the smooth endoplasmic reticulum. The disappearance of the glycogen granules from the liver following $CuSO_4$ and MD treatment may indicate a drug-specific effect, since 2, 4 D treatment didn't influence the distribution and density of glycogen granules in carp liver even after exposure of half a year (Benedeczky *et al.* 1984).

In general, the impoverishment of the parenchymal cells with respect to cell organelles can be observed following $CuSO_4$-MD treatment. At the same time, there is a striking increase in the amount of rough endoplasmic reticulum in

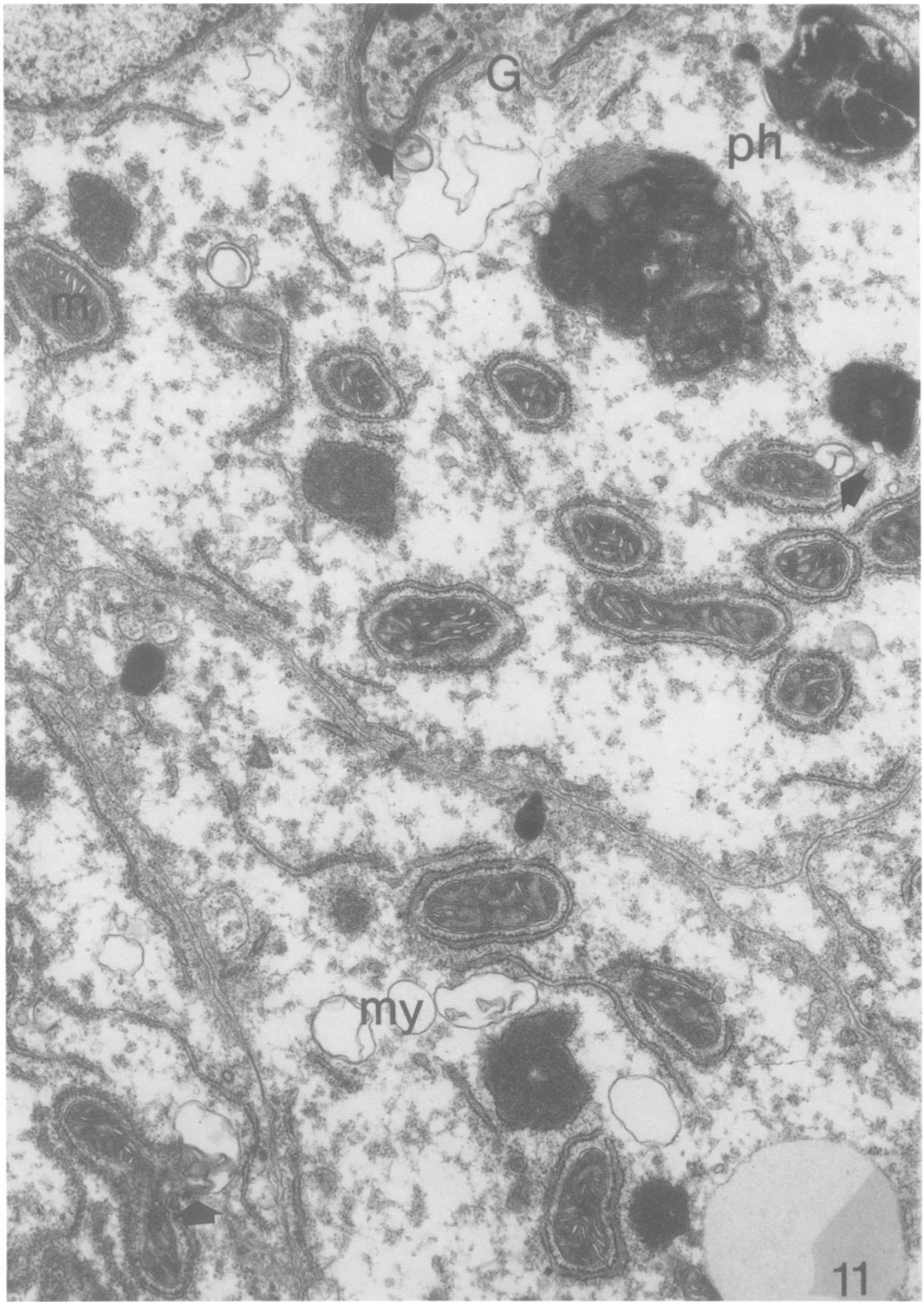

Fig. 11. Electron micrograph of carp liver, two weeks after $CuSO_4$ + MD treatment. The electron transparent cytoplasma of the parenchyma cells is poor in cell organelles, free ribosomes and glycogen are absent. The Golgi area (G) has strikingly collapsed, mitochondria (m) contracted. Several myelin figures (my) can be seen originated from Golgi, mitochondria, and endoplasmic reticulum (arrows) the increased endocytosis. 19,000×

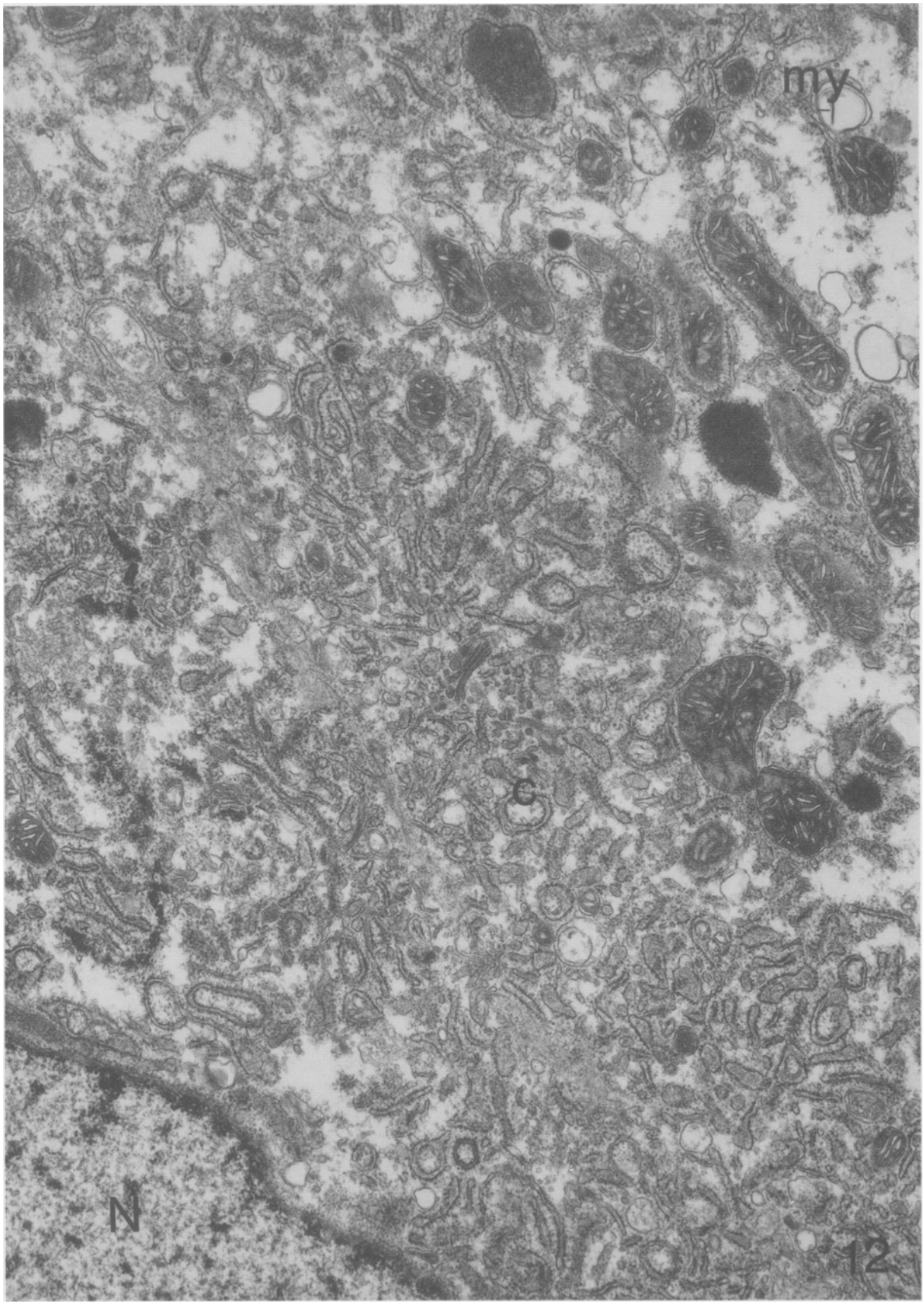

Fig. 12. A great number of rough endoplasmic reticulum cisternae (c) accumulated in some hepatocytes after treatment with $CuSO_4$ + MD. my = myelin figures, N = nucleus. 19,000×

certain cells (Figure 12), mainly in the perinuclear zone. This, however, does not consist of tubular elements, but rather a vesicular or cysternal arrangement is predominant, which is well known to be the consequence of a lower energy level (Schaff and Lapis 1979). The fine structure of the mitochondria in these cells is relatively well preserved, but their matrix is strikingly electron dense and they are generally small and elongated, in shape.

Myelin figures connected to the external membrane of the mitochondria were observed, indicating membrane decomposition. The development of myelin figures was also detected in associations with the membrane of the rough endoplasmic reticulum as well as the sheaths of the Golgi apparatus. Isolated myelin figures are observable in great numbers in the matrix of the hyaloplasm as well. The accumulation of the fine granular, highly dense substance was detectable in the cysternae of some strongly dilated rough endoplasmic reticulum. In addition to the clear type parenchymal cells, hepatocytes with dark cytoplasms also developed in lower number. Phagosomes (Figure 11) of varying shape, size and density were observable quite frequently in the hepatocytes of the treated animals. All these ultrastructural changes unambiguously indicate extensive damage of the protein synthesizing and energy-supplying cell organelles.

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